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TESTING OF EXPERIMENTAL COMPOUNDS FOR EFFICACY AGAINST *LEISHMANIA*

Annual Report

by
William L. Hanson, Ph.D., Virginia B. Waits, B.S.,
and Willie L. Chapman, Jr., D.V.M.

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A total of 82 compounds were studied in the primary visceral test system for suppressive activity against Leishmania donovani in golden hamsters. Thirty-two were noted to have some suppressive activity and 4 of these active compounds had activity greater than that of the reference compound, glucantime (Glucantime Index ranged from 2.62 to 14.18). Sinefungin was included among these highly active compounds.

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A total of 294 compounds were evaluated in the primary cutaneous test system for suppressive activity against cutaneous lesions resulting from Leishmania braziliensis panamensis in golden hamsters. Thirty-six of these had significant suppressive activity. Seven of these active compounds were 50-71% suppressive. Further testing of these compounds will be required for the calculation of Glucantime Indexes.

Following intracutaneous inoculations of hamsters near the base of the tail with 1.5×10^7 promastigotes of Leishmania braziliensis panamensis, a reasonably close correlation exists between lesion size and numbers of amastigotes during the first 6-9 weeks after infection in untreated hamsters. Following glucantime therapy on days 19-22 after infection, lesion size and numbers of amastigotes decrease concomitantly when evaluated at either 1 or 7 weeks after completion of therapy. Thus suppression of lesion size in this primary cutaneous test system is an excellent indicator of the suppressive effects of antileishmanial drugs especially during the first 6-9 weeks after infection.

Several purine analogues were noted to have antileishmanial activity against L. donovani. The activity of allopurinol <9 deazainosine < formycin B which is equivalent to formycin A. The activity of the latter were equal to or 2-4 fold greater than glucantime. Orally administered 9-deazainosine suppressed 96-99% of the amastigotes of L. donovani in the spleens and livers of squirrel monkeys indicating that the suppressive activity of this compound was equivalent to that of glucantime.

Liposome encapsulated amphotericin B (L-AmB) was highly active against L. donovani in hamsters and squirrel monkeys. A single administration of 1.5-11 mg of L-AmB/kg body wt suppressed greater than 99% of the amastigotes in the spleen and liver of hamsters and three administrations of 2 mg/kg of L-AmB/kg suppressed 90-95% of the amastigotes in the spleen and liver of squirrel monkeys with no deaths due to drug toxicity. Thus L-AmB was several hundred times as active as unencapsulated glucantime in the hamster and 50-60 times as active as unencapsulated glucantime in the squirrel monkey.

Two metabolites of WR06026 in hamsters were studied for antileishmanial activity in the hamster. One of these was highly suppressive while the other was inactive.

At least two preparations, namely 9-deazainosine and liposome encapsulated amphotericin B, emerge from these studies as candidates for clinical trials in human beings with visceral leishmaniasis. In addition, a third compound, sinefungin, deserves additional preclinical evaluations in laboratory animals.

FOREWORD

In conducting the research described in this report the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources. National Research Council. (DHEW Publication No. (NIH) 86-23, Revised 1985.)

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INTRODUCTION

The leishmaniases, the group of diseases caused by protozoan parasites of the family Trypanosomatidae, genus Leishmania, are widely distributed throughout the world and are found on every inhabited continent except Australia (Kinnamon et al., 1) occurring in such countries as Russia, China, India, Pakistan, Egypt, Sudan, Israel, Syria, Iran, Brazil, Venezuela, Panama, Mexico, Argentina, and many others. These diseases are transmitted by several species of phlebotomine flies and in most areas the leishmaniases are zoonoses with canines, rodents, or other mammals serving as reservoir hosts. These parasites are a significant health hazard to humans in these areas. Visceral leishmaniasis, the most severe type, is endemic in many areas where epidemics occur (TDR Publ, 7th Program Rpt., 2) with mortality reported to reach as high as 98 percent in untreated cases (Biagi, 3; Steck, 4). While it is difficult to obtain an accurate estimate of the number of human beings infected with the leishmaniases throughout the world, (TDR Publ, 7th Program Rpt., 2) at least one estimate indicates that at least 12 million persons have one of the different forms of the disease caused by infection with these parasites (Mahmoud and Warren, 5) and outbreaks involving additional thousands of persons occur periodically (Peters, 6 (TDR Publ, 7th Program Rpt., 2).

Infection with these parasites represents a significant health hazard to military personnel in many areas of the world. For example in World War II where troops were operating in an endemic area of the Persian Gulf 630 cases were reported in a three-month period (Most, 7). Subsequently during troop movements in another endemic area, 50 percent of certain Israeli forces experienced infections (Naggan et al., 8). In addition, although relatively few troops were involved, 10 to 45 cases per year were reported among U.S. troops in the Canal Zone (Walton et al., 9). Some mortality occurs but considerable loss occurs in duty time in infected individuals. For example it has been estimated that each individual having visceral leishmaniasis lost at least one year duty time (Most, 7) and in one instance in which 20 cases of cutaneous leishmaniasis occurred in troops in the Canal Zone, two man-years of duty time were lost (Walton et al., 9).

Efforts to control the leishmaniases have met with only limited success. The first line drugs currently available to treat the leishmaniasis are the pentavalent antimony compounds which are toxic with side effects which include vomiting, nausea, lethargy, and electrocardiographic changes and often repeated injections are required. In addition, these compounds are often not curative and evidence for antimony resistance among the Leishmania is increasing (Hanson, et al., 10). The current prospects for new drugs for the treatment of visceral

leishmaniasis are quite limited (WHO publication, 11). Drugs with proved efficacy in laboratory animals and which are currently undergoing pre-clinical or clinical studies are WR06026 and allopurinol riboside. Drugs or delivery systems in various stages of development and showing some promise are sinefungin, formycin B, miconazole, and liposomes. Formycin B has been observed recently in this laboratory to be extremely toxic in dogs and only marginally active and thus additional study of this compound is probably not warranted. Considerable work remains to be done before any of the others will be useful on a practical basis. Furthermore, the possibility that Leishmania already exists which are resistant to WR06026 (an 8-aminoquinoline) must be considered since these infections occur in areas of the world where 8-aminoquinolines have been used against malaria in humans. In this regard a strain of L. donovani from Kenya has been shown recently to be considerably more insensitive to antimony than a strain which has been in the laboratory for many years (Hanson et al., 10). and antimony resistant strains of L. donovani and L. braziliensis panamensis have been developed in this laboratory (Waits, et al., unpublished).

Because of the importance of leishmaniasis in human health and the need for improved and more satisfactory chemical compounds for consistent successful treatment of this disease, this project was initiated to test experimental compounds for efficacy against Leishmania donovani and L braziliensis panamensis infections in the golden hamster as the primary test systems and in non-human primates as a secondary test system. This is the first annual progress report for this project and this report covers the period 1 January 1985 through December 31, 1985. It describes the test procedures used and summarizes the results obtained. The test results obtained have been sent to appropriate officials at The Walter Reed Army Institute of Research as they became available during the contract year.

MATERIALS AND METHODS

I. Studies Involving Leishmania donovani

1. Primary Visceral Test System

A Khartoum strain of L. donovani (WR 378) was used in this part of the studies and the golden hamster (Mesocricetus auratus), 40-60 gm, served as the host animal. Suspensions of amastigotes for infection of experimental hamsters were prepared by grinding heavily infected hamster spleens in sterile saline in a Ten Broeck tissue grinder and diluting the suspensions so that 0.2 ml contained approximately 10×10^6 amastigotes. Each experimental hamster was infected via the intracardiac injection of 0.2 ml of the amastigote suspension.

The testing procedure used was that described by Stauber and his associates (12, 13, 14,) as modified by Hanson et al., (15). On day 3 following infection, hamsters were divided randomly into experimental groups consisting of a minimum of 6 animals per group, initial group weights were obtained, and administration of test compounds was initiated. Each compound was tested at 2 or 3 drug dosage levels dependent on the priority rating of the compound. Generally the test compounds with high priority ratings were studied initially via the intramuscular route (I.M.) at either 208, 52, or 13 MKD (milligrams/kilogram/day) while those compounds received with a routine or low priority rating were studied at 104 and 13 MKD only. Other drug dosage levels determined by the quantity of compound available or previous toxicity data were also used. The vehicle for the test compounds was 0.5% hydroxethylcellulose-0.1% Tween 80 (HEC-Tween). Each test group contained 6 hamsters and received one of the desired drug dosage levels. A control group of 6 to 8 hamsters received the 0.5% HEC-Tween vehicle only and the reference compound, glucantime, was given at 2 or 3 drug dosage levels (104, 13, and 3.25 MKD, or 104 and 13 MKD dosage levels (based on antimony content). All test compounds were routinely administered twice daily via the intramuscular route on days 3 through 6. Final group weights were obtained on all experimental hamsters on day 7 and all animals were killed, livers removed, weighed and liver impressions made for enumeration of amastigotes. Subsequently, the total number of parasites per liver was determined as described by Stauber (13).

In addition to recording body weight changes as a general indicator of toxicity of the test compounds, experimental hamsters were observed for such clinical signs of toxicity as nervous disorders, roughened hair coat, and sluggish activity. Deaths also were recorded. Weight loss of 15% or

greater and/or death of the animals was considered indicative of significant drug toxicity.

After determining the ratio of numbers of amastigotes/host cell nucleus, the weight of organ, and initial and final weights of the hamster the raw data was evaluated with a Televideo TS-802H microcomputer using a program which calculates percent weight change, total numbers of parasites, mean numbers of parasites/liver, percent parasite suppression and performs statistical analysis.

Additional information on the antileishmanial activity (estimation of potency) of each active compound was obtained by comparing the percent suppression of numbers of amastigotes it exhibits with the percent suppression observed with Glucantime, the reference compound. This comparative measure (referred to as the Glucantime Index or "G") was determined by the following formula:

$$\underset{(G)}{\text{Glucantime Index}} = \frac{SD_{90} \text{ for Glucantime}}{SD_{90} \text{ for new test compound}}$$

Drug dosage levels (MKD) required for a given degree of effect, such as 90% parasite suppression (SD_{90}) was estimated graphically from plots made on log-probit paper.

2. Special Cooperative Studies

As detailed in Memorandum for the Record, SGRD-UWM dated 7 June 1984, (Division of Experimental Therapeutics, WRAIR) a series of experiments were carried out during this project period on carefully selected compounds, many of which were either known to have in vivo antileishmanial activity or were suspected of having in vivo antileishmanial activity because of positive data from in vitro studies. The compounds studied are as follows: allopurinol, 9-deazainosine, 3-deazaguanosine, pentamidine, formycin A, formycin B, amphotericin B, ketaconazole, 6-mercaptopurine riboside, adenosine, nalidixic acid, novobiocin, aphidicolin, 4-mercapto-1H-pyrazolo - [3,4-d] pyrimidine, glycolate ester WR251815, WR248829, WR248830, WR250297, WR253563, WR253554, WR253549, WR253556, WR250678, and WR236391.

Procedures used for this experiment (parasite, 3 day infection, killing of hamsters, and enumeration of parasites) were similar to those used for the primary visceral screen. Routes of administration of the test compounds varied according to compound; the most efficacious route being used. Results were evaluated using a Televideo microcomputer program.

3. Liposome Studies in Hamsters

Neat and liposome encapsulated amphotericin B, formycin B, pentostam and glucantime were studied in L. donovani infected hamsters using procedures similar to those used for the primary visceral test system. All liposome preparations and neat amphotericin B were administered intracardially in a single injection, neat formycin B was

given orally in a single dose, and neat pentostam and glucantime were administered intramuscularly in a single injection. At the termination of the experiment, the hamsters were killed, spleens and livers were removed, and impression made for the enumeration of parasites as described for the primary test system. Results were evaluated using a Televideo microcomputer program as described in the preceding section.

4. WR06026 Analogue Studies in Hamsters

Experiments were carried out on two analogues of WR06026 (BK90014 and BL05884). The analogues as well as WR06026, glucantime, and vehicle only were administered to experimental hamsters twice a day for 4 days. The route of administration was either orally or intramuscularly as warranted for the test groups. Killing of hamsters, enumeration of parasites and data processing was the same as that used for the primary visceral test system.

5. Liposome Studies in Monkeys

Young adult squirrel monkeys (Saimiri sciureus) were used in this experiment. They were obtained from Charles River Research Primate Corporation, P. O. Box 416, Washington, N.Y. 11050. All except 2 were males. Two females were included to evaluate the toxicity and possible efficacy of "empty liposomes" because of the unavailability of additional males. Upon arrival in the laboratory the monkeys were checked for the presence of parasites, treated for intestinal nematodes and each was skin tested in the right eyelid with 1×10^6 killed promastigotes of L. donovani to determine previous contact with Leishmania. All monkeys were skin test negative.

Monkeys were infected with approximately 10×10^7 amastigotes of L. donovani per kg body weight obtained by grinding heavily infected hamster spleens in a Ten Broeck tissue grinder and diluting this homogenate with saline to a final concentration of 10×10^7 amastigotes/ml. Each monkey was infected IV based upon weight (i.e. a monkey weighing one killogram received 1.0 ml or 10×10^7 amastigotes).

On day 17 post infection, treatment was begun using liposome encapsulated amphotericin B, neat amphotericin B, glucantime, "empty liposomes" or saline (vehicle control). The treatment regimen varied from a single injection (saline and liposome encapsulated amphotericin B) every three days (neat amphotericin B; liposome encapsulated amphotericin B, or once a day for 7 days (glucantime). All preparations were administered via the intravenous route except glucantime which was given via the intramuscular route.

Blood samples were obtained from each monkey prior to infection, prior to initiation of treatment, as death occurred when possible, and from survivors when the experiment was terminated (Day 27 post infection). A blood sample was submitted for hematology and the remainder allowed to clot, serum collected, and stored at-80C, and was forwarded to WRAIR for analysis. A complete necropsy was performed on all monkeys and samples from all major organs were fixed in 10% buffered formalin and processed for histopathology examination. Gross lesions were recorded at necropsy.

Numbers of amastigotes were quantitated from impressions of the liver, spleen, (as previously described for the hamster) and the number of amastigotes /1000 host cell nuclei was determined from bone marrow preparation of each monkey.

6. WR06026 Studies in Monkeys

Adult squirrel monkeys used in this experiment were obtained from South American Primates, Inc., Miami, Florida and from the Center for Disease Control, Atlanta, Georgia The monkeys were adults and of both sexes.

Acclimitization and skin testing for Leishmania were the same as that described in the preceding section. Each experimental group contained two males and one female.

Treatment was initiated on Day 17 post infection and continued for 10 days. Dosage levels of 120, 60, 15, 3.75, and 0.94 total mg/kg of WR06026 were used. Three monkeys received vehicle only as controls. Numbers of amastigotes in the liver, spleen, and bone marrow were determined and mean percent suppressions determined as described in the preceding section.

7. 9-deazainosine Studies in Monkeys

Young adult, male squirrel monkeys obtained from Charles River Research Primate Corporation were acclimitized and skin tested as previously described.

Treatment was initiated on day 17 post infection and administered once a day for seven consecutive days. 9-deazainosine was given orally to groups of three monkeys each at 1400 and 350 total mg/kg while glucantime was administered intramuscularly at 364 and 91 total mg/kg. Three monkeys received vehicle only via the intramuscular route. Blood samples were obtained from monkeys receiving vehicle only and 1400 mg/kg of 9-deazainosine prior to infection, prior to initiation of treatment, and at necropsy. Blood samples were collected and hematology assays performed. A complete necropsy of these 6 monkeys was performed and samples from selected organs fixed in buffered formalin and processed for histopathology examination. Gross lesions were recorded at necropsy. Numbers of amastigotes in the liver, spleen, and bone marrow were calculated and mean percent suppressions calculated as described in the preceding section.

II. Studies involving Leishmania braziliensis panamensis

1. Primary Cutaneous Test System

Leishmania braziliensis panamensis (strain WR 539) was used in these studies. Male golden hamsters, 40-60 grams, served as experimental hosts.

In preparation for infection and weekly during the experiment, the hair was clipped on the dorsal tail head and a commercial dipilatory agent applied to the area to remove the remaining hair. Each hamster was inoculated via the intradermal route with 1.5×10^7 promastigotes of L.

braziliensis panamensis near the base of the tail using a 0.25 ml glass syringe equipped with a 30 gauge x 1/2" needle. Each experimental group consisted of six hamsters. Initial body weights were obtained and administration of therapy, generally via the intramuscular route, was initiated on day 19 post infection, and continued through day 22 post infection. Glucantime was included at two dosage levels (208 and 52 mg Sb/kg/day) as the reference compound and a group of six hamsters received vehicle only (HEC-Tween). Test compounds were administered generally at 104 or 52 MKD.

Lesion areas of each experimental hamster was determined with the aid of a template made at WRAIR and calibrated according to the formula $r_1 r_2 \pi$ where r_1 is the major radius of the lesions and r_2 is the minor radius of the lesion. (Wilson et al., 16). The mean lesion areas of each experimental group was obtained and the percent suppression of lesion size calculated by comparing the mean lesion areas of the treated groups with that of the group receiving vehicle only with the aid of a computer program and a Televideo TS-802H microcomputer. Comparison of the suppressive activity of test compounds with that of the reference compound, Glucantime, was made from log probit plots and a Glucantime Index for active compounds was calculated as described in the preceding section. Toxicity of test compounds was determined as indicated in the primary visceral test system.

2. Confirmation of Validity of Cutaneous Test System

A total of 250 hamsters were inoculated with L. braziliensis panamensis as described for the primary cutaneous test system. Approximately one-half of these were maintained without chemotherapy and the hair was removed from the lesion area by the weekly application of a commercial depilatory agent. Three or four of these hamsters were killed each week for 14 weeks following infection and lesions measured, excised, weighed, ground in 0.9% saline in a Ten Broeck tissue grinder and the numbers of amastigotes quantitated using the procedure described by Hanson and Roberson (17). A group of 12 of these hamsters were selected at random and lesion measurements were made at weekly intervals for 20 weeks. Six of these hamsters were killed at 2 months after infection and an additional 6 were killed at 4 months after infection and spleens homogenized and cultured in Schneiders Medium (Hendricks et al., 18) to check for presence of Leishmania. The remainder of the untreated hamsters were held to determine the ultimate fate of the cutaneous lesions.

The other half of the hamsters used in this experiment were divided at random into 18 groups and each group was treated on days 19-22 with either vehicle only, glucantime administered twice daily at 52 mg/kg body wt/day (MKD), glucantime administered twice daily at 208 MKD, glucantime administered twice daily at 416 MKD, glucantime administered at a total dosage of 208 mg/kg in a single treatment, and glucantime administred at a total dosage of 832 MKD in a single treatment. Lesion area was determined from six hamsters from each group beginning one week after completion of treatment and continuing for 6 weeks. Three hamsters from each group were killed one week after completion of treatment and another 3 hamsters from each group were killed 7 weeks after completion of treatment and lesions measured, weighed, ground in saline, and the number of amastigotes counted as

described by Hanson and Roberson (17).

The mean numbers of amastigotes per lesion were calculated for the lesions of all experimental groups and the effect of drug treatment on numbers of amastigotes was determined by comparing the mean number in the vehicle group with that of the treated groups. The percent suppression of amastigotes resulting from drug treatment was calculated for each drug dosage level used.

3. Special Cooperative Studies

One experiment was conducted during this project period as a part of the joint effort at a more rational approach to antileishmanial drug studies (detailed in Memorandum for the Record, SGRD-UWM dated 7 June 1984). The procedures used were the same as that for the primary cutaneous test system with the exception of route and regimen. Several of the most promising antileishmanial compounds were selected from those studied as described in a previous section (Special Cooperative Studies, Visceral leishmaniasis). These were allopurinol, Formycin A, and Formycin B. These compounds were compared for activity when administered either orally or intramuscularly using a bid x 4 day regimen. Amphotericin B was given either intracardially or intraperitoneally in a single injection. Vehicle only and glucantime treated hamsters were included. Results were evaluated using a Televideo microcomputer program.

RESULTS

I. Studies Involving Leishmania donovani

1. Primary Visceral Test System

A total of 82 compounds were studied at various drug dosage levels for suppressive activity against L. donovani in the primary visceral test system. Thirty-two of these yielded significant suppressive activity at one or more dosage levels. Five of these active compounds were sufficiently suppressive to warrant the calculation of Glucantime Indexes. The activity of four of these was greater than that of glucantime (BK85510, G = 2.62; ZP23714, G = 7.88; ZP46833, G =6.61; BL10956 (Sinefungin), G = 14.18) and one was less active (BL07931, G = 0.40). Three of these most active compounds (BK85510, ZP23714, BL10956,) were not toxic while the remaining two were toxic. Twenty of the 82 compounds studied were toxic as indicated by death and/or greater than 15% loss of body weight.

2. Special Cooperative Studies

A total of 26 compounds was selected for these in vivo studies based on known previous in vitro activity, previous evidence of in vivo activity, or suspected of having in vivo activity because of several types of evidence. These studies included a comparison of the in vivo activity of glucantime, amphotericin B, pentamidine, ketaconzaole, a number of purine analogs, as well as a variety of other compounds against visceral leishmaniases in the hamster when administered via various routes. Glucantime Indexes (G) could be calculated for 15 of these compounds and the relative activity of the others could be ascertained with some accuracy. The antifungal agent, amphotericin B when administered intravenously was the most active of this group of compounds (G = 27.33). This compound was less active when administered via the intraperitoneal route (G = 2.54). The purine analog, formycin B, administered orally was the second most active of the compounds studied in this experiment (G = 3.13). When administered via the intramuscular route, the activity was lower (G = 1.36). Formycin A administered per os (G = 1.88) and pentamidine administered intramuscularly (G = 1.02) were the next in the order of descending activity. The next most active compounds were 3-deazaguanosine and 9-deazoinosine with G's of 0.38 and 0.36 respectively when administered orally. The other compounds studied including allopurinol had activity less than those mentioned above.

Of the purine analogs studied, the only one for which greater than 95% suppressive dosage did not result in toxicity was 9-deazainosine.

3. Liposome Studies in Hamsters

A comparison of the antileishmanial activity of liposome encapsulated glucantime, pentostam, formycin B, and amphotericin B with that of unencapsulated glucantime, pentostam, formycin B, and amphotericin B was done in hamsters infected with Leishmania donovani. Liposome encapsulation generally resulted in enhanced antileishmanial activity against amastigotes in both the spleen and liver. Liposome encapsulated glucantime was 800-900 times as active as the unencapsulated drug (Berman et al., 19) and liposome encapsulated amphotericin B was 2-5 times as active as unencapsulated amphotericin B (Berman et al., 19). Furthermore, liposome encapsulated amphotericin B was 300-700 times more active than unencapsulated glucantime. Therapeutically effective dosages of liposome encapsulated amphotericin B were not toxic.

4. WR06026 Analogue Studies in Hamsters

Similar suppressive activity was noted in hamsters receiving WR06026 and analogue BK99014 when administered by either the oral or intramuscular routes. Slightly less weight loss was noted in hamsters receiving this analogue as compared to those hamsters receiving similar dosage levels of WR06026. Thirty-two percent suppression was noted in hamsters receiving analogue BL05884 at 13 total mg/kg when administered via the intramuscular route. This analogue was not significantly active at 1.62 or .2 mg/kg administered intramuscularly or at any dosage level administered per os. Less weight loss was again noted in hamsters receiving the analogue than in hamsters receiving WR06026 by either route.

5. Liposome Studies in Monkeys

The results of this experiment have been presented by Berman et al., (19). Unencapsulated glucantime, the reference compound, when administered at a dosage level of 104 mg/kg/day (MKD) administered on days 17-23 after infection eliminated greater than 98% of the amastigotes of L. donovani in the spleen, liver, and bone marrow of infected monkeys but caused the death of 1 of 3 of the experimental monkeys. Unencapsulated amphotericin B administered at 2 MKD on days 17, 20 and 23 eliminated more than 95% of the parasites in the spleen and liver and greater than 50% of the amastigotes in the bone marrow but both monkeys receiving this dosage level died due to toxicity of the compound. Liposome encapsulated amphotericin B administered at a dosage of 2 MKD on days 17, 20, and 23 eliminated 90-95% of the amastigotes in the spleen and liver and approximately 69% of the parasites in the bone marrow without causing any deaths of the monkeys. When the dosage of liposome encapsulated amphotericin B was increased to 4 MKD on days 17, 20, and 23 greater than 98% of the parasites were eliminated but 1 of 3 monkeys died. A single treatment on day 17 with 4 MKD of liposome encapsulated amphotericin B did not cause the death of any monkey but eliminated only 71 - 90% of the amastigotes in the spleen and liver respectively and approximately 69% of the amastigotes in the bone marrow.

Empty liposomes appeared to have some suppressive effect. Additional studies will be necessary to verify this observation.

Hematologic and microscopic pathologic studies revealed no difference between the treated and untreated groups.

6. WR06026 Studies in Monkeys

WR06026 was highly suppressive in the livers of all monkeys receiving total dosage levels of 120, 60, or 15 mg/kg body wt. (100%, >99%, and 91% respectively). No parasite suppression was seen in monkeys receiving total dosages of 3.75 or 0.94 mg/kg. Parasites in the spleens of monkeys receiving a total dosage of 60 mg/kg of WR06026 were highly suppressed (100%)while parasites in spleens of those receiving a total of 15 mg/kg were approximately 50% suppressed. It is of some interest to note that the absence of the spleen had no apparent effect on the efficacy of WR06026 as determined from parasite numbers in the liver and bone marrow.

Numbers of amastigotes in the bone marrow of treated monkeys were not suppressed at any drug dosage level used. WR06026 was not suppressive in any organ studied at total dosages of 3.75 or 0.94 mg/kg.

7. 9-deazainosine Studies in Monkeys

9-deazoinosine was highly active in spleen, liver, and bone marrow at a total dosage of 1400 and 350 mg/kg. Dosage levels of 200 or 50 MKD of this compound administered for seven days eliminated 99% of the amastigotes in the liver and 96% of the parasites in the spleen. The activity of the lower dose of 9-deazainosine was approximately equivalent to 52-104 MKD of the reference compound glucantime. No weight loss was noted in any of the groups of monkeys. Details of these studies are presented by Berman et al., (20).

Hematology studies based on a complete blood count revealed no significant differences between the vehicle control group and those receiving 9-deazainosine. Based on the histopathology studies of selected major organs only two major differences was observed between the group receiving vehicle only and those receiving the highest dosage level of 9-deazainosine. Fatty livers were noted in all three monkeys receiving the high level of 9-deazainosine while only one of 3 receiving vehicle had any evidence of fatty change. Numerous granulomas were observed in the livers of the vehicle control group while granulomas were sparse in those monkeys receiving the high level of 9-deazainosine. Numerous amastigotes were observed in the granulomas of vehicle controls while the granulomas in those receiving 9-deazainosine contained very few amastigotes.

We interpret the fatty change in the livers of the 9-deazainosine treated group as indicative of drug toxicity.

II. Studies Involving Leishmania braziliensis panamensis

1. Primary Cutaneous Test System

A total of 294 compounds were studied during this project period. Thirty-six of these were significantly active at one or both dosage levels used. Seven of the 36 active compounds yielded 50%-71% suppression of lesion size. No Glucantime Indexes were calculated due to having a single

significant point for plotting of the curve or the absence of a low enough significant point for the reference compound, Glucantime. Sixty-three of the 294 compounds studied were significantly toxic as indicated by death of hamsters and/or greater than 15% loss of body weight.

2. Confirmation of Validity of Cutaneous Test System

Following intracutaneous inoculation of hamsters near the base of the tail with 1.5×10^7 promastigotes of Leishmania braziliensis panamensis, cutaneous lesions increase in size generally 1-6 weeks reaching a maximum mean area of approximately 112 mm^2 by 6-8 weeks after infection and the area generally remains approximately the same for the next 8-10 weeks. Approximately 14-16 weeks after infection, lesions on some hamsters are observed to decrease in size, and during the next 12-15 weeks lesions on most hamsters decrease in size leaving subcutaneous granulomas. Lesions persist indefinitely on some hamsters. The weight of the lesion correlates closely with the area of the lesion.

Quantitation of the amastigotes in lesions from hamsters killed at weekly intervals after infection revealed that the mean total numbers of amastigotes per lesion increased during the first 3 weeks with maximum mean numbers of approximately 3.5×10^7 at three weeks. A gradual decrease in numbers occurred during the next 4 weeks after which the mean numbers of amastigotes per lesion decreased sharply and by 14 weeks after infection the mean number per lesion had decreased to approximately 9.5×10^5. The numbers of amastigotes remained low subsequently.

Following treatment of hamsters with 416, 208 or 52 MKD of glucantime on days 19-22 after infection the lesion size is decreased approximately 89%, 82% and 51% respectively when measured one week after completion of treatment. When measured 7 weeks after completion of treatment, the lesion suppression at these dosage levels was approximately 67%, 67% and 24% respectively. The numbers of amastigotes in lesions of hamsters treated with 416, 208, or 52 MKD glucantime on days 19-22 were decreased approximately 99%, 99%, and 97% respectively when counted one week after completion of treatment and were decreased approximately 99%, 94%, and 79% when counted 7 weeks after completion of treatment.

A reasonably close correlation exists between lesion size and numbers of amastigotes during the first 6-9 weeks after infection in the untreated hamster. In addition, following drug therapy on days 19-22 after infection, lesion size and numbers of amastigotes decrease concomitantly when quantitated at one or 7 weeks after treatment. Thus suppression of lesion size is an excellent indicator of the suppressive effects of antileishmanial drugs on the numbers of amastigotes in the lesions during the first 6-9 weeks after infection.

3. Special Cooperative Studies

Amphotericin B was only marginally active against L.b. panamensis when administered intravenously at the highest dosage used (3.25 mg/kg body wt in a single injection). Formycin B, total dosage of 260 mg/kg body wt., was significantly active (61% suppression) when administered

intramuscularly but was toxic at this dosage level. This compound was not active when administered orally and amphotericin B was not active when administered via the intraperitoneal route.

Allopurinol and formycin A were not significantly active when administered via either the oral or intramuscular routes. Allopurinol was toxic at 1664 total mg/kg when given intramuscularly. All hamsters receiving 1040 total mg/kg of formycin A orally died before completion of the experiment while significant toxicity was noted in hamsters receiving 52 total mg/kg of this compound via the intramuscular route.

III. Data Processing

During this project year in collaboration with officials at WRAIR, a new system for processing data was written, installed, revised and verified for both the L. donovani and L. braziliensis test systems. A new IBM-PC XT microcomputer was purchased with a DOS operating system which is compatable with WRAIR's VAX and the IBM-PC used by the COTR. There are several advantages of this new system over the old one which operated on CPM.

Since the IBM computer has a maximum record length of 220 bytes (twice that of the Televideo previously used) all pertinent data on a given test compound in any one experiment can be compiled into one record (line). This pertinent information contains bottle number, experiment number, drug route, drug regimen, test system, animal species used, parasite species used, julian date of infection, dosage levels used, percent weight change at each dosage level, number of amastigotes (or lesion size) of each animal for each dosage, parasite suppression of each dosage level, standard deviation, and Glucantime index.

The ability to compile this information into one record permits the establishment of a database which is able to retrieve any desired information on a given compound in a matter of minutes and display the data to the viewer. In collaboration with The Department of Experimental Therapeutics, WRAIR, during the last eleven years, the worlds largest data base on the experimental chemotherapy of leishmaniasis has been generated (approximately 5,000 compounds in the L. donovani system alone representing over 1 million bytes of data). Quick retrieval of the data is advantageous for WRAIR, the COTR, and this laboratory. Data can also be sorted using this system by date, bottle number, percent suppression, or Glucantime index.

Another advantage of this new computer is the increased memory capability (512 KB as compared to 64KB of the Televideo). This expanded memory allows for the storage of the large data base of test systems generated over the last 11 years and provides additional memory for the constant updating of newly acquired data .

The new programs written for this system by Major Patrick McGreevy, WRAIR, analyze the data using linear and non-linear regression. Glucantime Indexes are calculated using SD_{50} values rather than the SD_{90} values used with the Televideo system. By lowering the SD value, more promising compounds are brought to the attention of the COTR in the form of Glucantime Indexes.

The results reported herein were generated on the old Televideo System since most of the year was spent in developing and testing the new system. Since 1 January 1986, all data has been processed with the new IBM system. The data generated during the previous eleven years for the *L. donovani* test system has been rekeyed into the new system, verified for accuracy, and placed in a master database both at WRAIR and in this laboratory.

In addition, a new communications program was obtained (Hayes Smar modem) that allows better and easier user access for the transmittal of data and test protocols from this laboratory directly to the COTR and eventually directly into the VAX master files for future retrieval.

DISCUSSION

Since current means of therapy for the leishmaniases are not satisfactory for several reasons, these studies were conducted to identify new and better compounds with significant potential for consideration for future use in the treatment of human beings infected with protozoan parasites of the genus Leishmania. Several significant developments have been forthcoming from these efforts.

First, the results from the primary visceral test system have identified at least 3 new compounds with antileishmanial activity greater than the currently used antimonial, glucantime. These compounds probably warrant further study to determine their future potential. In addition, we have verified the considerable antileishmanial activity of sinefungin which has been reported by others to be active against L. donovani (Neal 21).

The Special Cooperative Studies resulted in a number of significant accomplishments. First, a study of the in vivo activity of several purine analogs gave a ranking of activity of allopurinol < 9-deazainosine < formycin A which is comparable to formycin B. The ranking of activity recorded for these compounds in vivo in the hamster model is similar to that obtained by Berman (20) against L. major in vitro in human monocyte-derived macrophages. Since these two models have obtained similar data on the antileishmanial activity of this group of compounds using two different species of Leishmania, one in vivo and the other in vitro, Berman et al. (20) have suggested that the activity of the purines can be assessed appropriately with these two models.

Another significant development from this series of experiments is the finding that 9-deazainosine was active against L. donovani in the hamster and that this compound was probably worthy of secondary testing in a monkey model available in this laboratory (Madindou et al., 22).

The followup studies of 9-deazainosine in the squirrel monkey were of considerable significance. This compound was at least as active and probably more active in the monkey than glucantime which is currently used in the treatment of visceral leishmaniasis in human beings. There was some suggestion that 9-deazainosine may have some toxic effects on the liver of the squirrel monkey. The advantage of 9-deazainosine over glucantime is that the former can be administered orally whereas the latter must be administered parenterally. Berman et al. (20) have suggested that consideration should be given to the development of 9-deazainosine as an oral treatment for human visceral leishmaniasis. The potential of this compound for use against antimony resistant Leishmania should also be

considered.

The marked enhancement of the antileishmanial activity of glucantime by encapsulation into liposomes previously reported (Alving et al., 23) has been confirmed in hamsters. Of considerably more importance however, are the observations in these studies that liposome encapsulated amphotericin B is highly active against L. donovani in both hamsters and squirrel monkeys. Unencapsulated amphotericin B is also active but therapeutic dosages may be toxic. However therapeutic dosages of liposome encapsulated amphotericin B which are highly efficacious against L. donovani in hamsters and monkeys have been shown previously not to be toxic to the kidneys nor other organs in human beings (Berman et al. 19). Liposome encapsulated amphotericin B is 60 times more active in the squirrel monkey than unencapsulated Sb (glucantime). Berman et al. (19) have suggested that the demonstration of high rodent and monkey efficacy with a clinical formulation of liposome encapsulated amphotericin B suggests that it should be considered for clinical trials in humans suffering from visceral leishmaniasis. This preparation will also have considerable potential for the treatment of antimony resistant Leishmania in human beings.

Available evidence regarding the highly active, antileishmanial drug WR06026, suggests that metabolites of this compound may be the effective antileishmanial agent(s). This evidence includes the considerably greater antileishmanial activity of this compound in hamsters than in mice or monkeys, and the greater antileishmanial activity of this compound is greater in the liver than in the spleen or bone marrow of the hamster. In view of this, metabolites of WR06026 are being identified and isolated and when possible synthesized by officials at WRAIR and are being tested for in vivo antileishmanial activity in this laboratory. One of these (BK90014) was noted to have suppressive activity similar to that of the parent compound while another (BL05884) was much less active. These studies are as yet in the very early stages but the results obtained thus far indicate that this is a very worthwhile endeavor and subsequent studies should yield highly significant results.

The relationship between lesion area and density of amastigotes in cutaneous leishmaniasis as documented here is, to our knowledge, the first information of this type available. Several significant points regarding these studies would appear to warrant further discussion. First, the observation that lesion area and numbers of amastigotes increase concomitantly during the early stages of the infection (1-3 weeks after infection), remain relatively constant for the next 4 weeks, and subsequently the numbers of amastigotes decrease sharply while lesion area generally remains unchanged has some practical implications. For example, in culturing lesions for the presence of parasites as a part of routine diagnostic procedures, the data suggest that older lesions may yield fewer positive cultures. Second, the observations that following chemotherapy during the early stages of the infection (days 19-22 after infection) the lesion area and the number of amastigotes are suppressed concomitantly verifies that evaluation of lesion area represents a valid assessment of the antileishmanial activity of the drug. Thus the primary cutaneous test system currently being used in this laboratory for the assessment of the antileishmanial activity of test compounds is valid.

Although considerable effort was devoted to identifying new compounds with suppressive activity against cutaneous lesions caused by L.b. panamensis, limited progress has been made in this area. This reflects the difficulty of successful chemotherapy of cutaneous leishmaniasis. This is emphasized by the fact that several compounds which were active against L. donovani had little or no activity against L.b. panamensis. The identification in the primary cutaneous test system of several new compounds with significant suppressive activity is promising. In addition, the observation that amphotericin B as well as formycin B (both of which are active against L. donovani) also have suppressive activity against L.b. panamensis suggests that a compound can be found that may be active against both species of Leishmania.

CONCLUSIONS

1. The primary visceral and primary cutaneous test systems are useful and valid in identifying new compounds with antileishmanial activity against Leishmania donovani and Leishmania braziliensis panamensis.

2. Leishmania donovani infected hamsters is a valid model for the evaluation of the antileishmanial activity of purines.

3. Based on the antileishmanial activity of 9-deazaninosine in hamsters and squirrel monkeys, infected with Leishmania donovani this compound appears to be a primary candidate for clinical trials in human beings with visceral leishmaniasis.

4. Based on the antileishmanial activity of liposome encapsulated amphotericin B in hamsters and squirrel monkeys infected with Leishmania donovani at a therapeutic dosage not toxic in human beings suggests this preparation as another primary candidate for clinical trials in human beings with visceral leishmaniasis.

RECOMMENDATIONS

1. Continue the primary visceral and cutaneous test systems at a moderate level to identify new classes of compounds with antileishmanial activity.

2. Increase activity in endeavors such as the Special Cooperative Studies project and select compounds for *in vivo* testing which show considerable promise in various types of *in vitro* studies. Also included should be selected compounds currently used in humans for other infectious diseases.

3. Continue to study *in vivo* the metabolic products of WR06026 as they can be identified and synthesized by officials at WRAIR.

4. Perform secondary testing of especially promising compounds in the squirrel monkey as a part of the logical sequence in antileishmanial drug development.

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